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Domains of the Human Immunodeficiency Virus Type 1 Matrix and gp41 Cytoplasmic Tail Required for Envelope Incorporation into Virions

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We recently demonstrated that a single amino acid substitution in matrix residue 12 (12LE) or 30 (30LE) blocks the incorporation of human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins into virions and that this block can be reversed by pseudotyping with heterologous retroviral envelope glycoproteins with short cytoplasmic tails or by truncating the cytoplasmic tail of HIV-1 transmembrane glycoprotein gp41 by 104 or 144 amino acids. In this study, we mapped the domain of the gp41 cytoplasmic tail responsible for the block to incorporation into virions by introducing a series of eight truncation mutations that eliminated 23 to 93 amino acids from the C terminus of gp41. We found that incorporation into virions of a HIV-1 envelope glycoprotein with a deletion of 23, 30, 51, or 56 residues from the C terminus of gp41 is specifically blocked by the 12LE matrix mutation, whereas truncations of greater than 93 amino acids reverse this defect. To elucidate the role of matrix residue 12 in this process, we introduced a number of additional single amino acid substitutions at matrix positions 12 and 13. Charged substitutions at residue 12 blocked envelope incorporation and virus infectivity, whereas more subtle amino acid substitutions resulted in a spectrum of envelope incorporation defects. To characterize further the role of matrix in envelope incorporation into virions, we obtained and analyzed second-site revertants to two different matrix residue 12 mutations. A Val→Ile substitution at matrix amino acid 34 compensated for the effects of both amino acid 12 mutations, suggesting that matrix residues 12 and 34 interact during the incorporation of HIV-1 envelope glycoproteins into nascent virions.

The matrix (MA) domain of the human immunodeficiency virus type 1 (HIV-1) Gag protein, which forms the N-terminal portion of the Gag precursor $Pr55^{Gag}$, has been postulated to serve a variety of functions in the virus life cycle. These include roles in virus assembly (6, 11, 18, 52, 60), targeting of $Pr55^{Gag}$ to the plasma membrane (52, 68, 69), virus entry (15, 65), nuclear localization of the viral preintegration complex (4, 25, 59), and envelope (Env) glycoprotein incorporation into virus particles (7, 13, 66). In virions, MA is localized just inside the lipid bilayer of the viral envelope (22) and is attached to the lipid bilayer by a bipartite membrane-binding domain composed of a covalently attached myristic acid moiety and a highly basic sequence near the N terminus of MA (63, 69).

Incorporation of viral Env glycoproteins during virus budding is an essential step in the formation of infectious virions. In HIV-1, the Env glycoproteins are incorporated as a complex of surface Env glycoprotein gp120 and transmembrane (TM) glycoprotein gp41. The gp120-gp41 complex mediates binding between the virion and the major HIV receptor CD4 and catalyzes a post-CD4 binding membrane fusion reaction that delivers the viral nucleocapsid into the host cell cytoplasm (for a review, see reference 14).

Details of the process by which Env glycoproteins are incorporated into retroviral particles are poorly understood. The close association between retroviral MA proteins and the lipid bilayers of the viral envelope and the host cell plasma membrane has encouraged speculation for more than a decade that an interaction might take place between MA and the cytoplasmic tail of the TM Env protein during the budding process. In the alphaviruses, such an interaction has been demonstrated between the viral capsid protein and the cytoplasmic tail of the E2 glycoprotein (45, 56, 57). However, conflicting data have been obtained with respect to a potential interaction between retroviral MA and TM proteins. Evidence in support of an interaction between retroviral MA and TM glycoproteins comes from a variety of studies. (i) The Rous sarcoma virus MA and Env glycoproteins could be chemically cross-linked (21). (ii) Single and multiple amino acid substitutions and deletions in HIV-1 and Mason-Pfizer monkey virus MA blocked Env glycoprotein incorporation (7, 13, 48, 66). (iii) In some studies, deletion of sequences in the cytoplasmic tail of HIV-1 gp41 blocked Env incorporation into virions (8, 67). (iv) Mutations in the cytoplasmic domain of the murine leukemia virus TM protein were reported to reduce Env incorporation into virions (23, 24). (v) HIV-1 MA facilitated the incorporation of HIV-1 Env into visna virus–HIV-1 chimeric particles (7). (vi) HIV-1 Env, specifically, sequences within the cytoplasmic tail of gp41, directed virus budding to the basolateral surface of polarized epithelial cells (35, 44).

Evidence against an interaction between the TM cytoplasmic tail and MA during Env incorporation also exists. Extensive deletions in the TM cytoplasmic tails of Rous sarcoma virus (46) and, in some studies, HIV-1 (19, 61) did not block Env incorporation into virions. Furthermore, a variety of heterologous viral Env glycoproteins (20, 33, 36, 53) and cellular glycoproteins (2) can be incorporated into HIV-1 virions.

We recently demonstrated (13) that a Leu→Glu substitution at MA residue 12 (12LE) or 30 (30LE) blocks the incorporation of HIV-1 Env glycoproteins into virus particles. The 12LE and 30LE MA mutations, however, did not affect the incorpo-

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ration of heterologous retroviral Env glycoproteins with short cytoplasmic tails. Furthermore, the phenotype of the 12LE MA mutation could be reversed by truncating the cytoplasmic tail of HIV-1 gp41 by 104 or 144 amino acids. These results indicated that an interaction between MA and the gp41 cytoplasmic tail might be required to direct the incorporation of Env glycoproteins with long cytoplasmic tails into HIV-1 virions. Subsequent to the publication of these results, others reported similar findings (38).

To define the domain(s) in the cytoplasmic domain of gp41 required for the 12LE-imposed block to Env incorporation, we introduced a number of additional truncation mutations spanning the gp41 cytoplasmic tail. Here we report on the effects of these mutations on Env incorporation into HIV-1 virions containing either wild-type or 12LE mutant MA. We also examined the specificity of MA residue 12 mutations by introducing a series of nonconservative and conservative substitutions into MA residues 12 and 13 and analyzing the effects of these changes on Env incorporation and virus infectivity. To better understand the role of MA in Env incorporation and further define the residues in MA involved in this process, we obtained and analyzed viral revertants that escape the Env incorporation defect imposed by MA residue 12 mutations.

MATERIALS AND METHODS

Site-directed mutagenesis. Mutagenesis of the sequence encoding the gp41 cytoplasmic tail was performed by subcloning the 540-bp *Bam*HI-*Kpn*I fragment (pNL4-3 nucleotide positions 8465 to 9005 [43]) spanning the 3′ portion of the *env* gene from HIV-1 Env expression vector pHenv (16) into M13mp18. Oligonucleotide-directed mutagenesis was then performed essentially by the method of Kunkle et al. (32), with a uracil-containing template and T4 DNA polymerase (New England BioLabs) at 37°C for 2 h. The nomenclature used for the gp41 C-terminal tail truncation mutations is as follows: CTdel-23 introduces a stop codon 23 amino acids from the C terminus of gp41, CTdel-30 introduces a stop 30 amino acids from the C terminus of gp41, etc. The following stop codon mutations were introduced by using the indicated oligonucleotides: CTdel-23, 5′-AAGTAGTATAAGGAGCT-3′; CTdel-30, 5′-ACGGGACATAAAGGGT TAT-3′; CTdel-51, 5′-GGAGTCAGTAACTAAAG-3′; CTdel-56, 5′-ATCTCC TATAGTATTGG-3′; CTdel-63, 5′-AAGCCCTCTAATATTGG-3′; CTdel-74, 5′-GGATTGTGTAACTTCTG-3′; CTdel-83, 5′-GAGAGACTAACTCTTGA-3′; CTdel-93, 5′-AGCCTGTGACTCTTCAG-3′.

Following mutagenesis, the 420-bp *Bam*HI-*Xho*I fragment spanning the C terminus of the *env* gene (pNL4-3 nucleotides 8465 to 8885 [43]) was recloned into pNL4-3 (1) or the pNL4-3 derivative containing the 12LE MA mutation (13). The *Bam*HI-*Xho*I fragment was sequenced in its entirety after cloning into pNL4-3 or pNL4-3MA/12LE.

None of these Env mutations disrupted Rev function, as determined by wild-type levels of Gag expression and virus production. One mutation (CTdel-89), which introduced a stop codon 89 amino acids from the C terminus of gp41, appeared to disrupt Rev function, since virus production with this mutant was severely impaired and the defect could be overcome by providing Rev in *trans* (data not shown). Because of this apparent Rev defect, the CTdel-89 mutant was not analyzed further. The CTdel-104 and CTdel-144 mutations, which were kindly provided by V. Bosch (Heidelberg, Germany), have been described previously (13, 61).

The amino acid 12, 13, and 34 mutations were introduced as described previously (18) by using the following oligonucleotides: 12LI, 5′-GGGGAGAAAT TGATAAATG-3′; 12LY, 5′-GGGAGAATATGATAAATG-3′; 12LW, 5′-GG GAGAATGGGATAAATG-3′; 12LD, 5′-GGGGAGAAGATGATAAATG-3′; 12LR, 5′-GGGGAGAACGAGATAAAT-3′; 12LK, 5′-GGGGAGAAAAAGA TAAAT-3′; 13DL, 5′-GAGAATTACTTAAATGGC-3′; 34VI, 5′-AACATAT AATCTGGGCAAG-3′; 34VE, 5′-AACATATAGAGTGGGCAAG-3′. To reduce the probability of primary-site reversions during T-cell line infections, all MA mutations were introduced by simultaneously changing at least two nucleotides.

Transfections and infections. The HeLa, HeLa T4 (37), CEM(12D-7) (50), and CD4-LTR-β-gal (MAGI) (29) cell lines were maintained as previously described (12, 29). Transfection of HeLa and HeLa T4 cells was performed with the calcium phosphate precipitation method, and CEM(12D-7) cells were transfected by the DEAE-dextran procedure as previously described (18). Syncytium formation assays (10) were performed as previously described (16), except that HeLa T4 cells were plated at 3.5×10^5 cells per well in six-well dishes and transfected with 6 μg of plasmid DNA per well. Infection of CEM(12D-7) and MAGI cells was performed as previously described (12, 13). Reverse transcriptase (RT) assays were performed as previously reported (11).

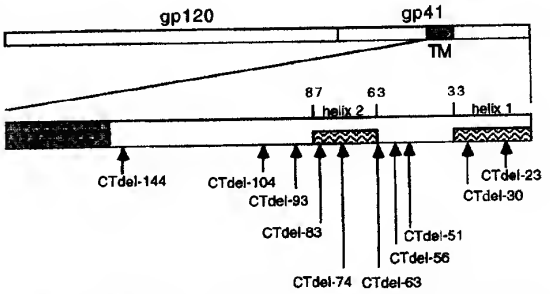

FIG. 1. Mutagenesis of the cytoplasmic domain of HIV-1 gp41. The top bar represents HIV-1 Env glycoproteins gp120 and gp41. The shaded box indicates the position of the gp41 membrane-spanning sequence; the boxes with wavy lines represent the positions of predicted α-helical stretches. The arrows indicate the positions of the C-terminal truncation mutations.

Cell and virus labeling and radioimmunoprecipitation analyses. Methods used in this study for metabolic labeling of transfected HeLa cells, preparation of cell lysates, pelleting of labeled virions in an ultracentrifuge, and immunoprecipitation of cell- and virion-associated proteins with AIDS patient sera have been described previously (12, 13, 62).

Molecular cloning of 12LE and 12LY revertant MA sequences. Virus was harvested from 12LE- and 12LY-transfected CEM(12D-7) cultures at the peak of RT activity and used to infect fresh CEM(12D-7) cells. At the peak of RT activity in the infected cultures, Hirt DNA (27) was prepared as follows. One million cells were washed once in phosphate-buffered saline and resuspended in 1 ml of 10 mM ETDA–100 mM Tris (pH 7.5); 10% sodium dodecyl sulfate (64 μl) was added, and the tubes were incubated on ice for 30 min. NaCl (3 M; 530 μl) was then added, and the tubes were incubated overnight at 4°C. The tubes were spun in an Eppendorf microcentrifuge for 15 min at 4°C, 200 μg of proteinase K was added, and samples were incubated at 37°C for 1 h. The DNAs were then extracted twice with a 1:1 mixture of phenol and chloroform, ethanol precipitated, and digested with RNase A. A 1.2-kbp fragment spanning the MA coding region was PCR amplified from the Hirt DNA. The primers used were as follows: positive, 5′-CTGACATCGAGCTTTCTACA-3′; negative, 5′-ATAGG TGGATTATGTGTCATC-3′. The positive- and negative-sense primers bound in the vicinity of pNL4-3 nucleotide positions (43) 340 and 1540, respectively. Thirty cycles of PCR were performed with approximately 10 ng of Hirt DNA under the following conditions: 58°C for 30 s, 94°C for 45 s, and 72°C for 3 min. Amplified DNA was digested with *Bgl*II and *Sph*I (pNL4-3 nucleotide positions 680 and 1440, respectively) (43). The *Bgl*II-*Sph*I fragments were cloned into the *Bam*HI and *Sph*I sites of pUC19 and sequenced in their entirety. Following sequencing, *Bss*HII-*Sph*I fragments from pUC19 clones were exchanged for the *Bss*HII-*Sph*I fragment of pNL4-3 (nucleotide positions 710 to 1440, respectively [43]) to generate the 12LE/34VI and 12LY/34VI double mutants.

RESULTS

Effects of gp41 cytoplasmic tail truncations on Env incorporation into virions containing wild-type or 12LE mutant MA. We previously determined that a Leu→Glu substitution at HIV-1 MA amino acid 12 (12LE) or 30 (30LE) blocks HIV-1 Env incorporation into virions and that truncation of the cytoplasmic tail of HIV-1 gp41 by 104 or 144 amino acids reverses the Env incorporation block (13). To investigate further the role of the cytoplasmic domain of gp41 in Env incorporation into virions containing wild-type or mutant MA, we used site-directed mutagenesis to introduce a number of additional termination codon mutations into the gp41 cytoplasmic tail (see Materials and Methods). These mutations resulted in truncation of the C terminus of gp41 by 23, 30, 51, 56, 63, 74, 83, or 93 amino acids. The locations of these truncation mutations with respect to the membrane-spanning domain and the two predicted α-helical domains in the gp41 cytoplasmic tail (58) are depicted in Fig. 1. The positions of the previously described truncation mutations CTdel-104 and CTdel-144 (13, 61; kindly provided by V. Bosch) are also indicated.

The gp41 cytoplasmic domain truncation mutations were introduced into pNL4-3 (1) containing either wild-type or

TABLE 1. Relative infectivity of double mutants bearing the 12LE MA and gp41 cytoplasmic domain truncation mutations

Virus[a]	gp41 truncation (no. of amino acids)	MA	Relative no. of IU/ml[b]	
			Assay 1	Assay 2
NL4-3	None	WT[c]	100	100
NL4-3/12LE	None	12LE	<1	<1
NL4-3(CTdel-23)	23	WT	18	29
NL4-3/12LE(CTdel-23)	23	12LE	<1	<1
NL4-3(CTdel-30)	30	WT	8	7
NL4-3/12LE(CTdel-30)	30	12LE	<1	<1
NL4-3(CTdel-51)	51	WT	18	33
NL4-3/12LE(CTdel-51)	51	12LE	<1	<1
NL4-3(CTdel-56)	56	WT	12	9
NL4-3/12LE(CTdel-56)	56	12LE	<1	<1
NL4-3(CTdel-63)	63	WT	<1	<1
NL4-3/12LE(CTdel-63)	63	12LE	<1	<1
NL4-3(CTdel-74)	74	WT	<1	<1
NL4-3/12LE(CTdel-74)	74	12LE	<1	<1
NL4-3(CTdel-83)	83	WT	<1	<1
NL4-3/12LE(CTdel-83)	83	12LE	<1	<1
NL4-3(CTdel-93)	93	WT	15	14
NL4-3/12LE(CTdel-93)	93	12LE	4	2
NL4-3(CTdel-104)	104	WT	91	87
NL4-3/12LE(CTdel-104)	104	12LE	114	64
NL4-3(CTdel-144)	144	WT	104	76
NL4-3/12LE(CTdel-144)	144	12LE	105	76
None (mock infection)			<1	<1

[a] HeLa cells were transfected with the indicated HIV-1 molecular clones. Two days posttransfection, virus supernatants were harvested, filtered, normalized for RT activity, and used to infect MAGI cells. Two days postinfection, cells were fixed, stained, and scored as previously described (13, 29).

[b] The data shown are the numbers of infectious units for the virus preparations relative to the wild type (NL4-3). Typical NL4-3 titers were approximately 7×10^3 IU/ml.

[c] WT, wild type.

12LE mutant MA. The resulting molecular clones were used to transfect HeLa cells, and virus pools were harvested, normalized for RT activity, and used to infect MAGI cells (29). The relative infectivities, measured by scoring the numbers of blue cells after infection, fixing, and staining (29), are presented in Table 1. Truncation of the C terminus of gp41 by 23, 30, 51, or 56 amino acids (CTdel-23, -30, -51, and -56) resulted in a 5- to 10-fold reduction in infectivity when the Env mutant was expressed in the context of wild-type MA. When the CTdel-23, -30, -51, or -56 truncation mutation was expressed in the context of the 12LE MA mutation, infectivity was abolished. Introduction of stop codons within predicted α-helix 2 of gp41 (CTdel-63, -74, and -83) completely blocked virus infectivity in the context of either wild-type or 12LE mutant MA. The infectivity of virions containing wild-type MA and a 93-amino-acid truncation of the C terminus of gp41 (CTdel-93) was reduced approximately sevenfold relative to that of wild-type NL4-3; the infectivity of double mutants containing both the 12LE MA mutation and the CTdel-93 gp41 truncation was reduced approximately 30-fold. As reported previously (13), the CTdel-104 and CTdel-144 mutations had no significant effect on virus infectivity, even in the context of the 12LE mutant MA.

The infectivity data presented in Table 1 suggest that the 12LE MA mutation blocks the virion incorporation of the CTdel-23, -30, -51, and -56 truncated Env glycoproteins and that the introduction of stop codons within predicted α-helix 2 of the gp41 cytoplasmic tail additionally may block Env incorporation in the context of wild-type MA. To directly assess Env incorporation of the truncated Env glycoproteins into virions containing either wild-type or mutant MA, HeLa cells transfected with the indicated molecular clones were metabolically labeled with [^{35}S]Cys, virions were pelleted in an ultracentrifuge, and cell- and virion-associated lysates were immunoprecipitated with AIDS patient sera (Fig. 2).

In the context of wild-type MA, the CTdel-23, -30, -51, and -56 truncations did not significantly affect Env incorporation into virions (Fig. 2A, top right panel). In contrast, truncations within predicted α-helix 2 of the gp41 cytoplasmic tail (CTdel-63, -74, and -83) resulted in the formation of virions with little or no detectable gp120 (Fig. 2A and B, top right panels). Mutant Env proteins containing truncations N terminal to predicted α-helix 2 (CTdel-93, -104, and -144) were incorporated into virions (Fig. 2B, top right panel).

In contrast to the results obtained with wild-type MA (Fig. 2A and B, top panels), virion incorporation of CTdel-23, -30, -51, and -56 truncation mutants was blocked in the presence of the 12LE MA mutation (Fig. 2A, lower right panel). As observed with wild-type MA, Env mutants containing truncations within predicted α-helix 2 were not stably incorporated into virions containing the 12LE MA mutation. Furthermore, gp41 truncations N terminal to predicted α-helix 2 relieved the 12LE-imposed Env incorporation block observed with wild-type HIV-1 Env and with the CTdel-23, -30, -51, and -56 truncated Env glycoproteins (Fig. 2B, lower right panel).

The 12LE MA mutation blocks virion incorporation of unprocessed Env precursor gp160. Because gp41 was not readily apparent in our immunoprecipitations of virion-associated material (because of comigration of gp41 with the p41 Gag processing intermediate [41]), we cannot definitively distinguish between a block to Env incorporation into virions and simply enhanced shedding of gp120 from virions after incorporation. In long exposures of the gels presented in Fig. 2, we observed that the increased mobility of the truncated gp41 mutants resulted in clear separation of gp41 from p41. Whereas the truncated TM glycoprotein could be clearly visualized in CTdel-23, -30, -51, and -56 virions containing wild-type MA, it was absent in virions containing the 12LE MA mutation (data not shown). These results suggested that the absence of detectable gp120 in 12LE mutant virions reflected an incorporation defect rather than enhanced gp120 shedding.

To address this issue further, we determined whether the 12LE mutation abrogates Env incorporation into virions when gp120 shedding from virions cannot occur. We utilized a previously described HIV-1 Env cleavage mutant (120.518A) which cannot undergo processing of gp160 to gp120 and gp41 (16). The 120.518A mutation was introduced into pNL4-3 and the pNL4-3 derivative containing the 12LE MA mutation to create pNL4-3/120.518A and the double mutant pNL4-3/12LE/120.518A. Radioimmunoprecipitation analysis revealed gp160 in the 120.518A virions (which contain wild-type MA), whereas no gp160 was detected in the 12LE/120.518A double mutant virions (Fig. 3). Together, these data demonstrate that the 12LE MA mutation blocks HIV-1 Env incorporation into viri-

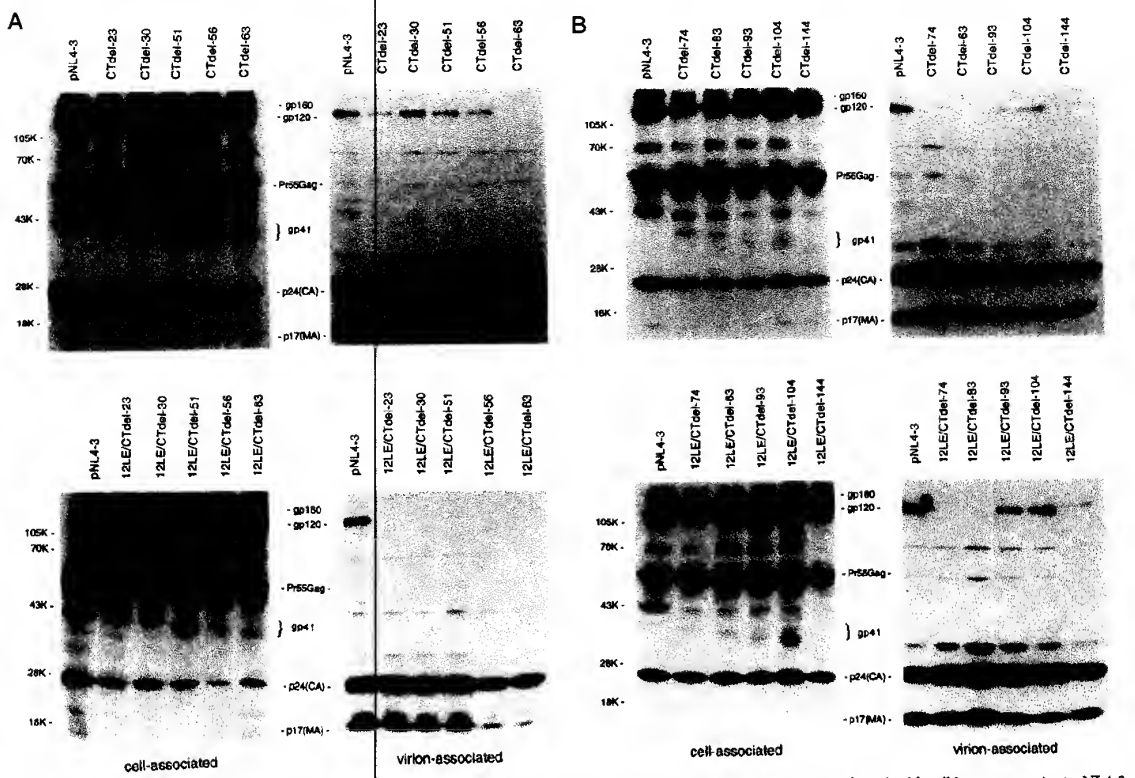

FIG. 2. Radioimmunoprecipitation analysis of CTdel mutants and 12LE-CTdel double mutants. HeLa cells were transfected with wild-type or mutant pNL4-3 molecular clones and metabolically labeled overnight with [^{35}S]Cys. Virion-associated material was pelleted in an ultracentrifuge; lysates derived from cell- and virion-associated material were immunoprecipitated with AIDS patient sera (see Materials and Methods). (A) CTdel-23, -30, -51, -56, and -63 mutants. (B) CTdel-74, -83, -93, -104, and -144 mutants. The top panels show CTdel gp41 mutants in pNL4-3 clones expressing wild-type MA; the lower panels show CTdel mutant clones expressing the 12LE MA mutation. Cell-associated proteins are on the left, and virion-associated proteins are on the right. The positions of Env precursor gp160, mature surface glycoprotein gp120, Gag precursor Pr55^{Gag}, TM glycoprotein gp41, p24(CA), and p17(MA) are indicated; the sizes of molecular mass markers are shown in kilodaltons (K).

ons, rather than affecting the stability of the noncovalent association of gp120 with gp41.

The 12LE MA mutation does not block functional Env expression at the cell surface. The data presented above indicate that the 12LE MA mutation specifically abrogates the incorporation of full-length HIV-1 Env and mutant Env proteins containing C-terminal truncations of 23, 30, 51, and 56 amino acids. Since several studies had raised the possibility that HIV-1 Gag and Env proteins may interact before budding occurs, possibly even before transport to the cell surface (35, 44), it seemed possible that the 12LE mutant Gag might impair the transport of Env glycoproteins with long cytoplasmic tails to the cell surface, where Env incorporation into budding particles occurs.

To assess the effect of the 12LE MA mutation on cell surface Env expression, we evaluated whether the 12LE MA mutation affects syncytium formation, a property of HIV-1 Env which depends upon functional Env expression at the plasma membrane (for a review, see reference 14). The CD4$^+$ HeLa cell line HeLa T4 (37), which is highly susceptible to HIV-1 Env-induced syncytium formation, was transfected in parallel with wild-type pNL4-3 or with pNL4-3 derivatives expressing 12LE MA mutant and/or cytoplasmic tail truncation mutant gp41 proteins. Two days posttransfection, cells were stained and syncytia were scored microscopically as described previously (16). The data obtained (Table 2) indicated that the 12LE MA mutation had no effect on the ability of the wild type or any of the truncated Env glycoproteins to induce syncytia. These results demonstrate that the 12LE MA mutation does not block functional expression of HIV-1 Env at the cell surface.

Interestingly, some of the Env truncations did affect syncytium formation. The longest truncations (CTdel-104 and CTdel-144) significantly enhanced fusion, as previously reported (61), whereas two truncations within predicted α-helix 2 of the gp41 cytoplasmic tail (CTdel-74 and -83) almost completely abolished syncytium formation.

Mutagenesis of MA amino acid 12. To evaluate the specificity of MA amino acid 12 mutations on Env incorporation, we introduced a number of other single amino acid substitutions into MA residue 12: Leu to Asp (12LD), Arg (12LR), Lys (12LK), Tyr (12LY), Trp (12LW), and Ile (12LI). Several of these, i.e., 12LD, 12LR, and 12LK, like the original 12LE mutation, were charged substitutions; others were more subtle. We also introduced an Asp→Leu substitution at MA residue 13 (13DL).

The single amino acid substitution mutations were intro-

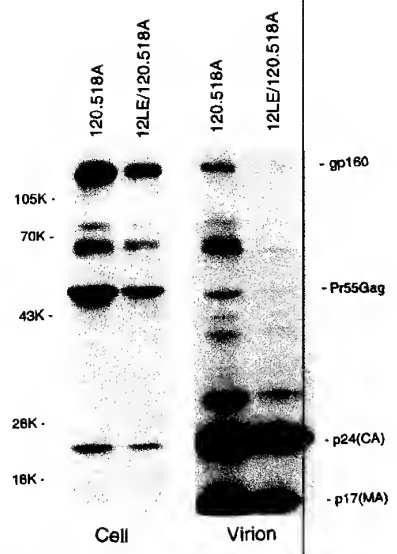

FIG. 3. Radioimmunoprecipitation analysis of unprocessed gp160 incorporation into wild-type and 12LE MA mutant virions. HeLa cells were transfected with a pNL4-3 clone expressing the 120.518A gp160 processing mutant protein (120.518A) or with the 12LE MA mutant expressing the 120.518A gp160 processing mutant protein (12LE/120.518A). Two days posttransfection, transfected cells were metabolically labeled overnight with [^{35}S]Cys; cell- and virion-associated proteins were immunoprecipitated with AIDS patient sera (see Materials and Methods). Cell-associated proteins are on the left, and virion-associated proteins are on the right. The positions of Env precursor gp160, Gag precursor $Pr55^{Gag}$, p24(CA), and p17(MA) are indicated; the sizes of molecular mass markers are shown in kilodaltons (K).

duced into the full-length molecular clone pNL4-3, virus preparations were generated in HeLa cells, and virus infectivity was analyzed in MAGI cells. The results indicated that the charged substitution mutations at MA residue 12, i.e., 12LE, 12LD, 12LR, and 12LK, almost completely blocked virus infectivity in this assay (data not shown). The neutral substitutions at residue 12, i.e., 12LW, 12LY, and 12LI, and the 13DL mutation resulted in 2- to 10-fold reductions in virus infectivity relative to the wild type.

The MAGI infectivity data suggested that charged substitution mutations at MA residue 12 block Env incorporation into virions, whereas the more subtle mutations lead to less severe reductions in Env incorporation. To directly assess the effects of the amino acid 12 and 13 substitutions on the levels of virion-associated gp120, cell- and virion-associated lysates were subjected to radioimmunoprecipitation analysis (Fig. 4). Consistent with the infectivity data, the charged substitution mutations (12LE, 12LR, 12LK, and 12LD) resulted in loss of detectable gp120 in virion-associated material. The 12LW, 12LY, 12LI, and 13DL mutations significantly decreased, but did not abolish, Env incorporation. Quantitation of the amount of gp120 relative to the amount of p24(CA) present in the virion-associated material with a PhosphorImager indicated that the 12LW, 12LY, 12LI, and 13DL changes decreased levels of virion-associated gp120 3- to 10-fold (data not shown).

The infectivity of HIV-1 residue 12 MA mutants in a T-cell line was assessed by transfecting CEM(12D-7) cells in parallel with wild-type pNL4-3 and monitoring virus production over time by RT assay (Fig. 5). No virus replication was detected in cultures transfected with the 12LE, 12LD, 12LR, or 12LK mutant molecular clone, corroborating the results of MAGI infectivity assays (data not shown) and the gp120 incorporation studies (Fig. 4). The 12LI, 12LW, and 12LY mutations delayed peak virus production in transfected CEM(12D-7) T-cell cultures 6 to 14 days relative to that of wild-type pNL4-3. The 13DL mutation delayed peak virus production 4 days relative to that of the wild type (data not shown).

Reversion of amino acid 12 mutations. To explore the possibility that second-site revertants of the 12LE MA mutant might arise following long-term passage of 12LE-infected cultures, a number of independent CEM(12D-7) transfections were carried out with pNL4-3 molecular clones containing the 12LE mutation (data not shown). In one experiment (Fig. 6A), substantial virus replication was detected at approximately 1 month posttransfection. To assess the replication kinetics of the virus derived from this 12LE-transfected culture, supernatant medium was harvested from pNL4-3- and 12LE-transfected cells at the peaks of RT activity and fresh CEM(12D-7) cells were infected with equivalent amounts (normalized for RT activity) of virus (Fig. 6B). The repassaged virus derived from the 12LE-transfected cultures replicated with kinetics that were delayed by only 6 days relative to the wild type,

TABLE 2. Relative syncytium formation induced by gp41 truncation mutants with wild-type or 12LE mutant MA

Molecular clone[a]	gp41 truncation (no. of amino acids)	MA	Relative syncytium formation[b]
pNL4-3	None	WT[c]	100
pNL4-3/12LE	None	12LE	99
pNL4-3(CTdel-23)	23	WT	134
pNL4-3/12LE(CTdel-23)	23	12LE	177
pNL4-3(CTdel-30)	30	WT	116
pNL4-3/12LE(CTdel-30)	30	12LE	128
pNL4-3(CTdel-51)	51	WT	125
pNL4-3/12LE(CTdel-51)	51	12LE	133
pNL4-3(CTdel-56)	56	WT	279
pNL4-3/12LE(CTdel-56)	56	12LE	292
pNL4-3(CTdel-63)	63	WT	121
pNL4-3/12LE(CTdel-63)	63	12LE	108
pNL4-3(CTdel-74)	74	WT	7
pNL4-3/12LE(CTdel-74)	74	12LE	10
pNL4-3(CTdel-83)	83	WT	11
pNL4-3/12LE(CTdel-83)	83	12LE	13
pNL4-3(CTdel-93)	93	WT	271
pNL4-3/12LE(CTdel-93)	93	12LE	278
pNL4-3(CTdel-104)	104	WT	611
pNL4-3/12LE(CTdel-104)	104	12LE	614
pNL4-3(CTdel-144)	144	WT	612
pNL4-3/12LE(CTdel-144)	144	12LE	645

[a] HeLa T4 cells (37) were transfected with the indicated molecular clones. Two days posttransfection, cells were stained and the number of syncytia was scored microscopically (Materials and Methods; reference 16).

[b] Data are presented as the number of syncytia obtained relative to the number obtained with wild-type pNL4-3 and are averages of at least three transfection assays.

[c] WT, wild type.

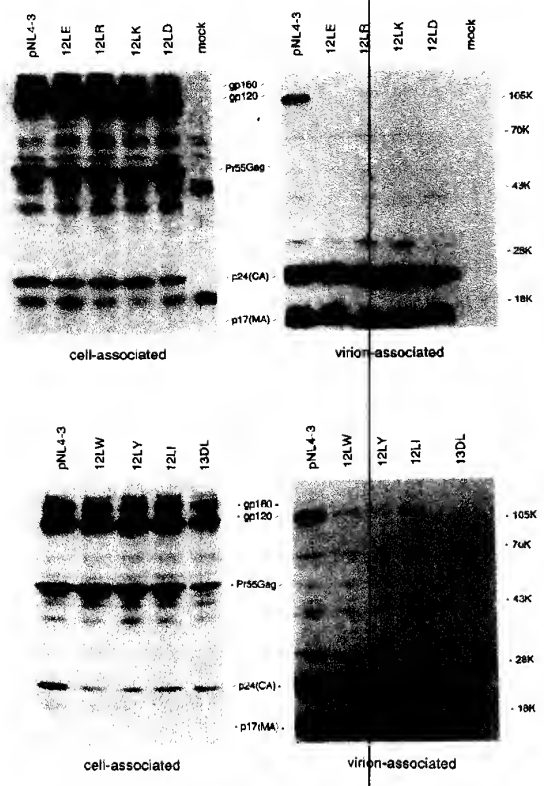

FIG. 4. Radioimmunoprecipitation analysis of MA amino acid 12 and 13 mutants. HeLa cells were transfected with wild-type pNL4-3 or with a pNL4-3 derivative containing the 12LE, 12LR, 12LK, 12LD, 12LW, 12LY, 12LI, or 13DL MA mutation. Two days posttransfection, transfected cells were metabolically labeled overnight with [^{35}S]Cys; cell- and virion-associated proteins were immunoprecipitated with AIDS patient sera (see Materials and Methods). Cell-associated proteins are on the left, and virion-associated proteins are on the right. The positions of Env precursor gp160, mature surface glycoprotein gp120, Gag precursor $Pr55^{Gag}$, p24(CA), and p17(MA) are indicated; the sizes of molecular mass markers are shown on the right in kilodaltons (K).

suggesting that viral revertants had emerged in the 12LE-transfected cultures.

A similar analysis was performed with the 12LY mutant. CEM(12D-7) cells were again transfected in parallel with pNL4-3 or the 12LY mutant (Fig. 6C). Virus was harvested at the peak of RT production, and fresh CEM(12D-7) cells were infected with the 12LY putative revertant virus in parallel with an equivalent amount of wild-type virus (Fig. 6D). The repassaged 12LY virus grew with kinetics very similar to that of the wild type.

A Val→Ile change at MA residue 34 is present in both 12LE and 12LY revertants. To identify second-site changes that might compensate for the effects of the 12LE and 12LY mutations on Env incorporation, Hirt supernatant DNAs were prepared at the peak of RT activity from CEM(12D-7) cells infected with the putative 12LE or 12LY revertant virus and PCR was performed to amplify the MA coding region (see Materials and Methods). The PCR-amplified DNA was then cloned into pUC19, and the entire MA coding region was sequenced. Sequencing data from six 12LE clones and two 12LY clones are presented in Fig. 7. Two of the six 12LE-derived clones (12LE/rev1 and -2) contained a primary-site reversion which restored the wild-type codon (Leu) at residue 12. Four of the six 12LE clones (12LE/rev3 to -6) maintained the 12LE mutation but acquired a single nucleotide substitution that resulted in a coding change (Val→Ile) at MA amino acid 34.

Interestingly, sequencing analysis of two 12LY revertant clones indicated that both (12LY/rev1 and -2) maintained the original 12LY mutation and acquired the same Val→Ile change at MA residue 34 seen in the 12LE revertant clones. No other coding changes were present in the sequenced portion of the *gag* gene in any of the 12LE or 12LY revertant clones.

The 34VI change compensates for the Env incorporation defect imposed by MA amino acid 12 mutations. The finding that a Val→Ile change at MA amino acid 34 (34VI) was present in both 12LE- and 12LY-derived clones suggested that the 34VI change might be responsible for the markedly improved replication kinetics of the repassaged 12LE and 12LY viruses. To analyze the effect of the 34VI change in the context of the 12LE or 12LY change, we constructed 12LE/34VI and 12LY/34VI double mutants by cloning the *Bss*HII-*Sph*I fragment from the 12LE/rev3 and 12LY/rev1 pUC19 subclones into pNL4-3. This strategy resulted in the generation of isogenic pNL4-3 derivatives that contained 12LE/34VI and 12LY/34VI double mutations. No other coding changes were introduced in this process.

To assess the effect of the 12LE/34VI or 12LY/34VI double mutation on the establishment of a productive infection, the CEM(12D-7) T-cell line was transfected in parallel with pNL4-3 or pNL4-3 derivatives containing the 12LE, 12LE/34VI (Fig. 6E), 12LY, and 12LY/34VI (Fig. 6F) mutations. In wild-type pNL4-3-transfected cultures, peak RT activity was evident at 8 days posttransfection. No virus replication was detected in 12LE-transfected cultures at any time during the course of this experiment. In a CEM(12D-7) culture transfected with the 12LE/34VI double mutant, peak virus replication occurred with a delay of only 4 days relative to the wild type. The replication kinetics of the 12LE/34VI double mutant relative to that of wild type closely resembled that of the

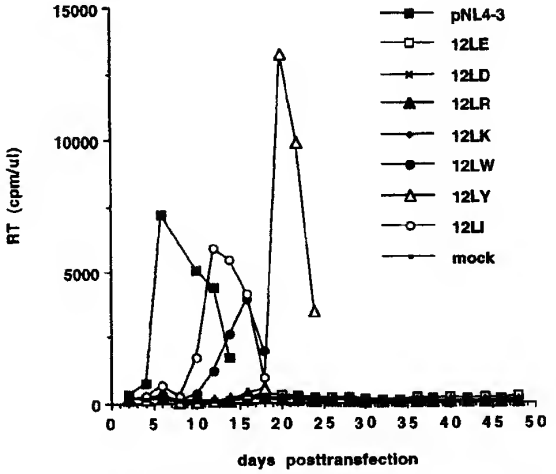

FIG. 5. Replication kinetics of MA amino acid 12 mutants in the CEM(12D-7) T-cell line. Cells were transfected in parallel with wild-type pNL4-3 or the MA amino acid 12 mutant derivatives and split 1:3 every 2 days. Aliquots were reserved at each time point for RT assays.

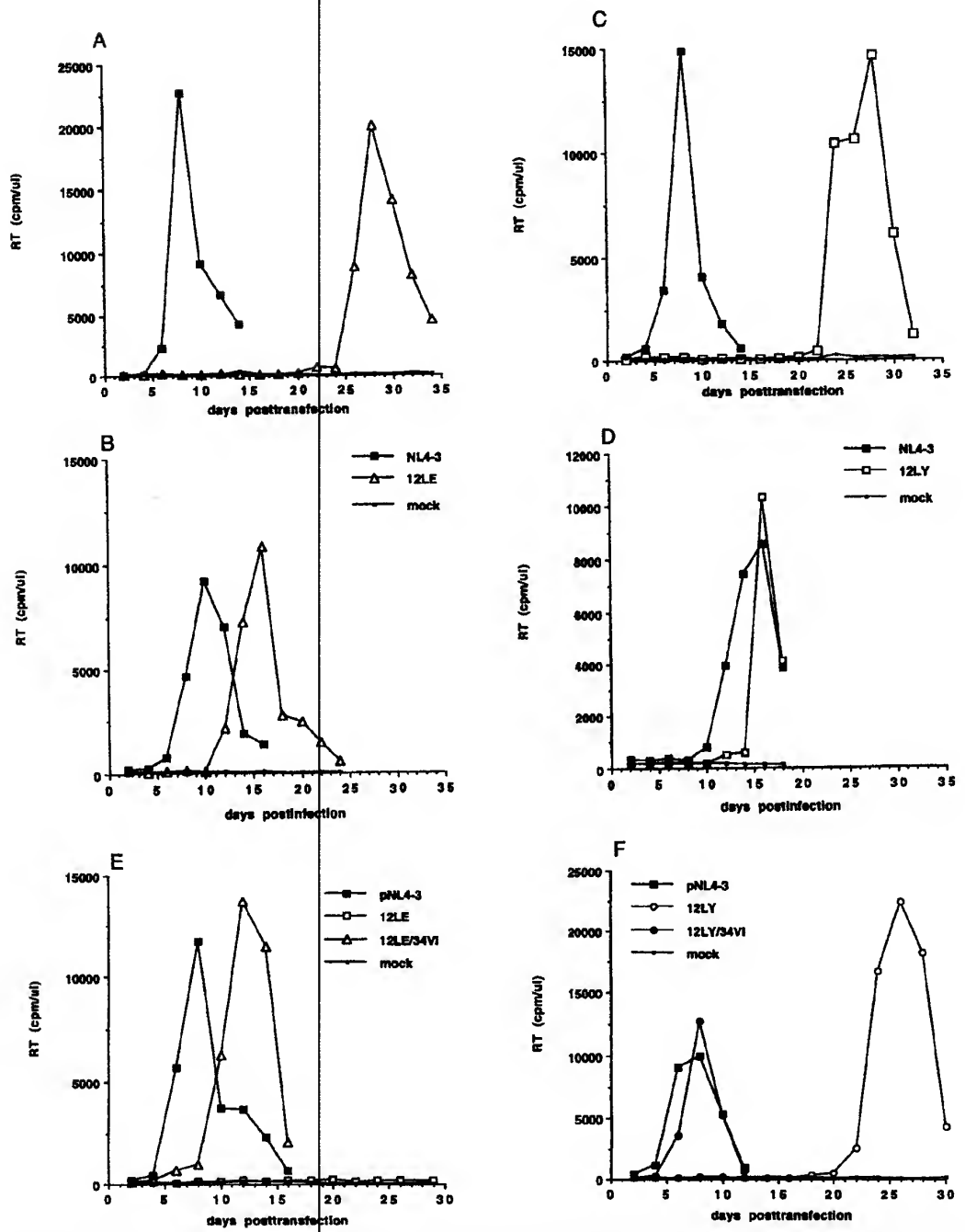

FIG. 6. Growth kinetics of 12LE and 12LY MA revertants and 12LE/34VI and 12LY/34VI double mutants. CEM(12D-7) cells were transfected with 12LE (A), infected with the putative 12LE revertant virus (B), transfected with 12LY (C), infected with the putative 12LY revertant virus (D), or transfected with the 12LE (E), 12LE/34VI (E), 12LY (F), or 12LY/34VI (F) MA mutant. Details concerning transfections and infections are provided in the text. Cells were split 1:3 every 2 days; aliquots were reserved at each time point for RT assays.

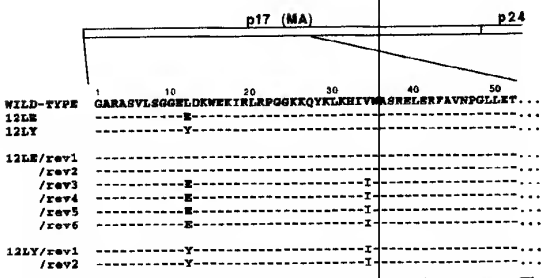

FIG. 7. Molecular cloning and sequencing of amino acid 12 revertants. The sequence of wild-type pNL4-3 MA (residues 1 to 52) is at the top in the single-letter amino acid code. Immediately below are the sequences of the 12LE and 12LY mutants, below which are indicated the amino acid sequences of six pUC19 12LE revertant clones and two 12LY pUC19 revertant clones. The single amino acid change at MA residue 34 (34VI) is shown (see Materials and Methods). Dashes indicate amino acid sequence identity with the wild type.

original uncloned 12LE revertant (Fig. 6B). Virus production in 12LY/34VI-transfected cultures peaked on day 8 posttransfection, whereas peak virus replication in 12LY-transfected cultures did not occur until 26 days posttransfection, undoubtedly recapitulating the emergence of revertant viral progeny.

The data presented in Fig. 6E and F suggest that the 34VI mutation at least partially reversed the Env incorporation block resulting from the amino acid 12 mutations. To analyze Env incorporation directly, HeLa cells were transfected with pNL4-3 or with a pNL4-3 derivative containing the 12LE/34VI or 12LY/34VI double mutation and metabolically labeled with [^{35}S]Cys 2 days posttransfection. Cell- and virion-associated material was prepared and immunoprecipitated with AIDS patient sera as described in Materials and Methods. The immunoprecipitation data indicate that the 12LE/34VI mutant virions (Fig. 8A) contained significantly more gp120 than did 12LE virions, which failed to incorporate detectable amounts of gp120 (Fig. 4) (13). Quantitation by PhosphorImager analysis of the gp120 band relative to p24 in the 12LE/34VI virion-associated lane of the immunoprecipitation shown in Fig. 8A indicates that 12LE/34VI virions contained approximately threefold less gp120 than did wild-type virions (data not shown). The Env incorporation in 12LY/34VI virions was indistinguishable from that of the wild type (Fig. 8B), in marked contrast to the approximately 10-fold-reduced Env incorporation observed with 12LY virions (Fig. 4). These results demonstrate that the 34VI change significantly enhanced Env incorporation in the context of either the 12LE or the 12LY mutation.

Analysis of the effect of MA amino acid 34 mutations on Env incorporation. The finding that viral revertants of both the 12LE and 12LY mutants acquired the same compensatory change, a Val→Ile substitution at MA amino acid 34, suggests that MA residue 34, like residues 12 and 30 (13), plays a role in Env incorporation into virions. To examine this possibility, site-directed mutagenesis was used to introduce two single amino acid substitutions at MA residue 34 in wild-type pNL4-3. The changes introduced were Val→Ile (34VI) and Val→Glu (34VE). Virus production in CEM(12D-7) cultures transfected with either wild-type pNL4-3 or the 34VI mutant peaked on day 8 posttransfection; peak virus production in 34VE-transfected cultures was delayed approximately 1 week relative to wild type (Fig. 9).

The virus replication data presented in Fig. 9 suggested that the 34VI mutation alone had no significant effect on Env incorporation into virions, whereas the 34VE mutation might cause an Env incorporation defect. To directly address this issue, we immunoprecipitated cell- and virion-associated material obtained from HeLa cells transfected with pNL4-3 or the residue 34 mutants. Consistent with the CEM(12D-7) infectivity data, the 34VI mutation had no detectable effect on Env incorporation, whereas the 34VE mutation resulted in virions which lacked detectable gp120 (Fig. 8B).

DISCUSSION

We previously demonstrated (13) that polar substitution mutations at positions 12 (12LE) and 30 (30LE) of HIV-1 MA

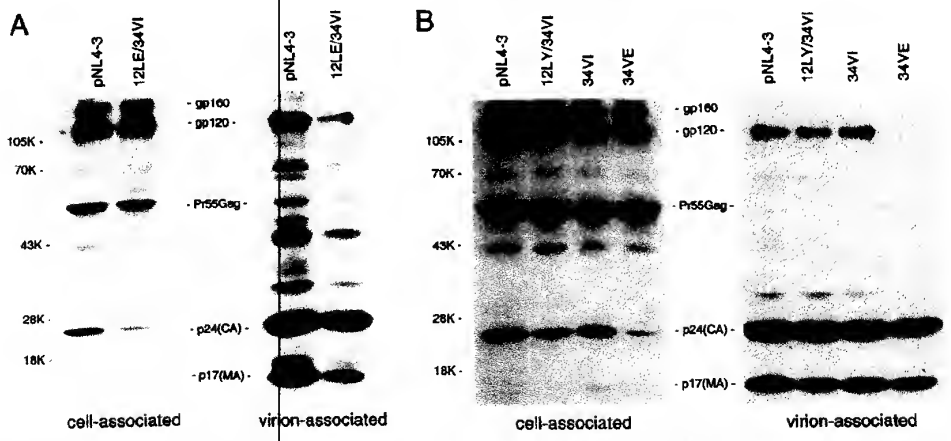

FIG. 8. Radioimmunoprecipitation analysis of the 12LE/34VI, 12LY/34VI, 34VI, and 34VE MA mutants. The wild type and the 12LE/34VI mutant (A) and the 12LY/34VI, 34VI, and 34VE mutants and the wild type (B) are compared. HeLa cells were transfected with a wild-type or mutant pNL4-3 molecular clone and metabolically labeled overnight with [^{35}S]Cys. Virion-associated material was pelleted in an ultracentrifuge; lysates derived from cell- and virion-associated material were immunoprecipitated with AIDS patient sera (see Materials and Methods). Cell-associated proteins are on the left, and virion-associated proteins are on the right. The positions of Env precursor gp160, mature surface glycoprotein gp120, Gag precursor Pr55^{Gag}, p24(CA), and p17(MA) are indicated; the sizes of molecular mass markers are shown on the left in kilodaltons (K).

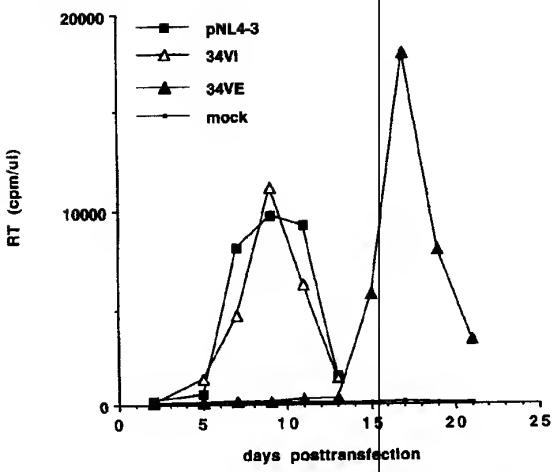

FIG. 9. Replication kinetics of MA amino acid 34 mutants. The CEM(12D-7) T-cell line was transfected in parallel with pNL4-3 or derivatives containing the 34VI or 34VE MA mutation. Cells were split 1:3 every 2 days; aliquots were reserved at each time point for RT assays.

block the incorporation of wild-type HIV-1 Env into virus particles. These mutations, however, did not affect the incorporation of two heterologous retroviral Env glycoproteins with short cytoplasmic tails: the amphotropic murine leukemia virus Env and a naturally truncated form of HIV-2 Env. Furthermore, truncation of a significant portion (104 or 144 amino acids) of the 150-amino-acid HIV-1 gp41 cytoplasmic tail reversed the Env incorporation block observed with the 12LE MA mutation. These results raised the possibility that sequences in the C-terminal 104 amino acids of gp41 might interact with MA to facilitate Env incorporation into virions.

The results of gp41 truncation analyses presented here indicate that the 12LE MA mutation blocks the incorporation of HIV-1 Env glycoproteins with cytoplasmic tail truncations of up to 56 amino acids from the gp41 C terminus. Truncation of 93 or more residues from the C terminus reverses the Env incorporation block imposed by the 12LE MA mutation. Levels of virion gp120 are markedly reduced by truncations of 63 to 83 amino acids from the gp41 C terminus, even in the presence of wild-type MA. This region corresponds to putative gp41 cytoplasmic tail α-helix 2 (58; Fig. 1). The fact that the incorporation of Env glycoproteins with truncations C terminal to predicted α-helix 2 is blocked by the 12LE MA mutation whereas truncations N terminal to α-helix 2 reverse the 12LE-imposed incorporation block, together with the finding that truncations within α-helix 2 significantly reduce levels of gp120 in virions containing wild-type MA, suggests that residues within or immediately adjacent to putative gp41 cytoplasmic tail α-helix 2 take part in an interaction with MA during Env incorporation. It is interesting that several truncations, i.e., CTdel-23, -30, -51, and -56, which do not markedly reduce levels of gp120 in virions containing wild-type MA (Fig. 2A) significantly reduce infectivity in MAGI cells (Table 1). As has been suggested previously (8, 19, 28, 34, 67), these results indicate that the gp41 cytoplasmic domain plays a role in virus infectivity, as well as in Env incorporation.

To determine whether the Env incorporation defect observed with the 12LE MA mutation (13) was specific for a Glu substitution, we introduced a number of additional changes at MA residue 12 and one change at position 13. Charged substitutions at MA residue 12 (12LR, 12LK, and 12LD), like the original 12LE mutation, resulted in particles that were devoid of detectable gp120 (Fig. 4). More subtle changes in residue 12 and a nonconservative (Asp→Leu) substitution at residue 13 reduced but did not block Env incorporation. These results indicate that the Env incorporation block initially observed with the 12LE mutant is not specific for a Glu substitution and that even very subtle changes at this position (e.g., 12LI) significantly reduce levels of virion gp120.

Long-term passage of CEM(12D-7) cultures transfected with the 12LE or 12LY mutant led to the emergence of viral revertants. This was apparently a rare event with the 12LE mutant, since only one transfection in six led to virus particle production in the transfected cultures (data not shown). A low reversion rate for 12LE might be anticipated from the severity of the 12LE-imposed defect. With the 12LY mutant, in which the initial Env incorporation and infectivity defects were less pronounced than in the 12LE mutant (Fig. 4), virus replication was consistently observed at around 1 month posttransfection in all experiments (Fig. 5 and 6F; data not shown). Interestingly, both 12LE and 12LY revertants acquired the same Val→Ile substitution at MA position 34 (34VI). Characterization of 12LE/34VI and 12LY/34VI double mutants demonstrated that the 34VI change significantly enhanced virus infectivity (Fig. 6E and F) and Env incorporation (Fig. 8) in the context of either position 12 change. These results suggest that residues 12 and 34 interact during Env incorporation and that MA amino acids 12, 30 (13), and 34 may form a gp41 binding domain. Involvement of MA residue 34 in Env incorporation is further suggested by the finding that a Val→Glu substitution at this position (34VE), in the absence of other MA changes, causes a very marked reduction in the level of virion gp120 (Fig. 8B). Examination of the proposed nuclear magnetic resonance-derived structure of MA (39, 40) indicates that residue 12 is located in MA helix I and residues 30 and 34 are located in MA helix II. The formation of a three-stranded mixed β-sheet involving residues 18 to 20, 21 to 25, and 94 to 96 with a turn around the Pro at residue 22 places residues 12, 30, and 34 close to each other in the tertiary structure of MA. Comparison of the replication kinetics of 12LE, 30LE, and 34VE (Fig. 5 and 9; reference 13) suggests that 34VE virions contain somewhat more gp120 than do 12LE or 30LE virions.

Several models could explain the mechanism by which the position 12 MA mutations block Env incorporation into virions. (i) A specific interaction between lentiviral Env glycoproteins and MA evolved to facilitate the incorporation of Env glycoproteins with long cytoplasmic tails into virions. Mutations within MA (e.g., at residue 12, 30, or 34) disrupt this interaction, thus blocking Env incorporation. This model is consistent with our data and with the observations that HIV-1 Env glycoproteins are not incorporated into MuLV virions (64), whereas retroviral Env glycoproteins with short cytoplasmic tails are readily incorporated into HIV-1 viral particles. (ii) MA-Env interaction is not required for virion incorporation of Env glycoproteins with long cytoplasmic domains, but the MA mutations that block Env incorporation do so by altering the conformation of MA (i.e., these mutations create an unfavorable interaction). We cannot exclude this possibility, although the creation of such unfavorable interactions would have to be highly specific for particular changes in MA, since most of the single and double amino acid substitutions we have analyzed do not block Env incorporation (11, 13, 15, 18). Furthermore, the observation that even very subtle changes in MA position 12 (for example, the 12LI change), which would be predicted to have little or no impact on overall MA conformation, sig-

nificantly reduce the level of gp120 in virions (Fig. 4) makes this model appear unlikely. (iii) MA mutations that block Env incorporation do so by preventing the stable expression of Env at the cell surface, where incorporation into budding virions occurs. This apparently does not occur, since the 12LE substitution has no effect on the ability of wild-type Env or any of the truncated Env mutants to induce syncytia, which depends upon functional cell surface Env expression.

Although the 12LE MA mutation does not appear to affect functional cell surface Env expression, some of the gp41 truncation mutations within putative α-helix 2 may affect Env stability, processing, or transport, as evidenced by the reduced ability of the CTdel-74 and -83 mutants to induce syncytia. These mutations might also affect the conformation of the extracellular domain of gp41, as studies with truncated simian immunodeficiency virus Env glycoproteins have suggested that cytoplasmic domain truncations can affect the conformation of the TM ectodomain (55). It seems likely that the CTdel-63 truncation mutation affects Env incorporation directly, rather than cell surface expression or gp120 shedding, since mutations affecting these properties also markedly reduce syncytium formation and the levels of cell-associated gp120 (17, 31). As demonstrated here, neither the amount of cell-associated gp120 (Fig. 2) nor syncytium formation (Table 2) is significantly affected by the CTdel-63 mutation. Definitive elucidation of the mechanism by which gp41 α-helix 2 truncations result in the formation of virions lacking gp120 requires a more rigorous analysis of the effect of these truncations on Env processing, transport, cell surface expression, and gp120-gp41 association.

Interestingly, several of the gp41 cytoplasmic tail truncations (in particular, CTdel-104 and -144) significantly increase HIV-1 Env-mediated syncytium formation (Table 2). Increased fusogenicity of truncated Env glycoproteins has been observed previously with HIV-1 (8, 9, 61), HIV-2 (42), simian immunodeficiency virus (49, 54, 70), Mason-Pfizer monkey virus (3), murine leukemia virus (47), and influenza virus (51), suggesting that sequences in the cytoplasmic domain of viral Env glycoproteins play a role in modulating membrane fusion.

The data presented in Table 1 indicate that the CTdel-104 and -144 truncation mutants are as infectious as wild-type HIV-1 in MAGI cells. However, in transfection and infection experiments with several T-cell lines [SupT1, CEM(12D-7), and H9], we were unable to establish a productive infection with any of the mutants containing short gp41 cytoplasmic tails (15). In contrast, most of the gp41 truncation mutants, including CTdel-104 and -144, readily established a productive infection in MT-4 cells (15, 61). These results indicate that productive infection of most T-cell lines requires sequences in the long cytoplasmic tail of gp41 and that cell type-specific differences exist with respect to the requirement for these sequences in the virus life cycle. Such cell type-specific differences are also evident from reports indicating that simian immunodeficiency virus variants with truncated TM cytoplasmic tails rapidly emerge upon passage in human cells, whereas in monkey cells the TM cytoplasmic tail remains full length (5, 26, 30). The observation of cell type-specific requirements for the gp41 cytoplasmic tail, together with our finding that the exact length of the gp41 truncation determines its phenotype (with respect to Env incorporation into virions with wild-type MA), in part reconciles apparent contradictions in the literature (see the introduction) concerning the role of the gp41 cytoplasmic tail in HIV-1 infection and Env incorporation (8, 19, 61, 67).

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